Environmental Health Perspectives
Vol. 9, pp. 283–294, 1974

Pharmacology of Fibrosis and Tissue Injury

by Milos Chvapil*

Methods controlling tissue fibrosis are classified into those *specifically* inhibiting various metabolic aspects of collagen *selectively* in the injured tissue (ascorbic acid deficiency, effect of agent chelating Fe^{2+}, proline analogs, lathyrogens). The most promising method seems to be the blocking of crosslinks formation among collagen molecules by β-aminopropionitrile, a competitive inhibitor of a crosslinking enzyme, lysyl oxidase. The second group of methods is called nonspecific, as they affect any stage of inflammatory process preceding the activation of fibroblasts. The importance of activated macrophages in the stimulation of fibroblast is discussed. Finally, a new concept is proposed, indicating the function of zinc ions in the control of the integrity of biomembrances, tissue reactivity to noxious agents. It is suggested that zinc may control NADPH dependent lipid peroxidation at the membrane level by inhibiting NADPH oxidase activity. The implication of these ideas to lung fibrosis induced by silica or asbestos is discussed.

I would like to focus attention on two topics of great importance to all of us who are involved in problems of fibrogenesis and tissue injury. The first topic deals with the pharmacological control of the abnormal deposition of collagen in forms of various fibrotic, cirrhotic, or sclerotic lesions. To this category belongs lung fibrosis after fibrogenic dusts. The second topic will deal with the role of various metals in the development of tissue injury with several implications related to metallic ions adjacent to the asbestos fiber.

Methods to Control Abnormal Accumulation of Collagen in Tissues

The extensive research material *(1–3)* indicates that there are basically two approaches to inhibit collagen synthesis and deposition. The first and obviously most desired possibility would be to inhibit collagen selectively (not the other tissue components) and specifically only in a tissue which has been injured and in which a fibroproliferative inflammation takes place. The second approach recognizes that abnormal collagen deposition is the final step of fibroproliferative inflammation and stresses that the pharmacological control of inflammatory chain reactions should begin with early stages before the fibroblast becomes involved (stimulated). Let us critically analyze the value of either method.

The methods of selective and specific inhibition of collagen are based on characteristic features of collagen synthesis and metabolism summarized in Figure 1.

Collagen synthesis follows the principles established for any other protein. There are, however, some unique steps in the life of collagen. Collagen is born on ribosomes where the polypeptide chain is growing. At this time, it contains only prolyl and lysyl residues and no hydroxyproline or hydroxylysine. Only when the chain reaches a certain length is it hydroxylated. Hydroxylation of some prolyl and lysyl residues by prolyl (lysyl) hydroxylase is a unique process characterizing collagen synthesis. The presence of OH of hydroxylysine is essential for the following glycosylation, which again is a

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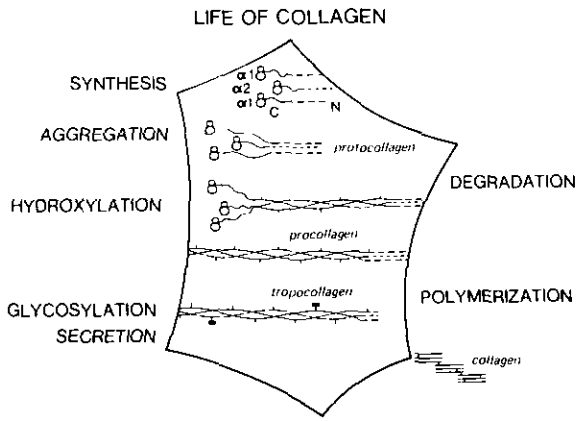

FIGURE 1. Schematic representation of principal steps in collagen metabolism. The sequence of events follows: Synthesis of subunits, polypeptide chains (α_1, α_2) with additional peptides (broken line), which control proper aggregation into procollagen. Hydroxylation (vertical bars) begins at the ribosome level and is completed after polypeptides are released from the ribosomes. Glycosylation of hydroxylysine hydroxyl groups by glucose or *gelactose-glucose enables secretion of the whole molecule* of triple helixed procollagen or tropocollagen (without additional peptides). Polymerization refers to the formation of covalent crosslinks. Rate of degradation is inversely related to the degree of polymerization of collagen structures.

prerequisite for the secretion of the whole molecule of tropocollagen out of the fibroblast. This process is slightly more complicated because, as you know, tropocollagen consists of three unidentical polypeptide chains forming a triple helix. A certain mechanism exists which enables a proper fitting of these subunits into a register to form the collagen molecule. Once collagen is secreted from the cell into the interstitial space, it ages. This process, also called maturation or polymerization, refers to the formation of the strong covalent crosslinks. The enzyme involved in the synthesis of these linkages is a lysyl oxidase. Finally, collagen dies and is degraded with the highest mortality in the early postnatal period. The younger and therefore more soluble collagen is, the faster it is degraded by tissue collagenases and, in further stages, possibly digested by other tissue proteases.

In this highly simplified review of the events in the life of collagen, two steps have attracted enormous attention of experimental workers aiming at selective and specific collagen inhibition, i.e., hydroxylation and polymerization.

Inhibition of Collagen Hydroxylation

Inhibition of collagen hydroxylation was chosen because it was found that the underhydroxylated and consequently underglycosylated collagen is not secreted at all or extruded at a slower rate by fibroblasts. Thus, it accumulates within the cell and, by still unknown mechanism, possibly by some form of negative feedback, it inhibits the synthesis of the whole collagen molecule. The inhibition of collagen hydroxylation was achieved by interfering with individual cofactors or cosubstrates of prolyl hydroxylase (Fig. 2) which needs for its function ascorbic acid, ferrous iron, α-ketoglutarate, and oxygen.

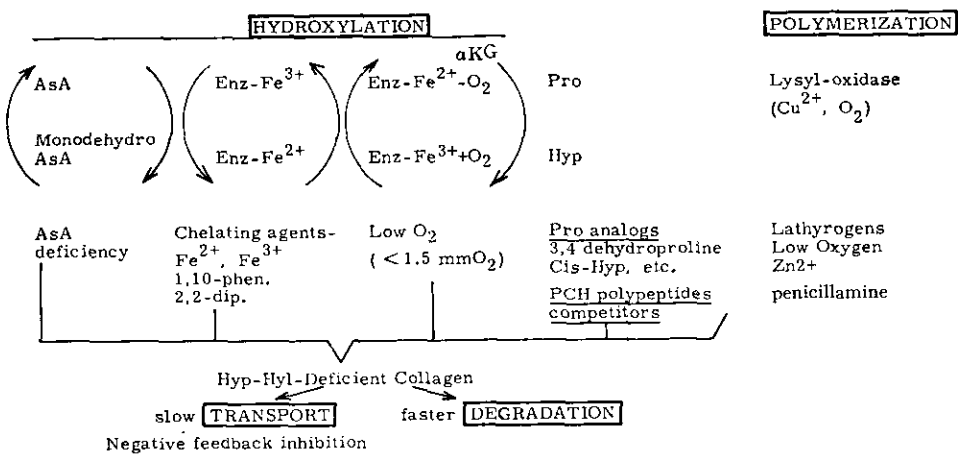

FIGURE 2. Methods of selective control of collagen metabolism. Asa=ascorbic acid; PCH=protocollagen proline hydroxylase; Pro=proline; Hyp=hydroxyproline.

There is considerable amount of information on the *in vitro* (tissue slices and fibroblasts in tissue cultures) *(1)* and *in vivo* effects of depletion of ascorbic acid, chelation or iron, low-oxygen tension on the extent of hydroxylation of collagen and/or on collagen accumulation, and synthesis in various models of fibroproliferative inflammation. In brief, in *in vitro* systems, interference with either of the above-mentioned cofactors of prolyl hydroxylase results in the synthesis of a crippled (under-hydroxylated and under-glycosylated) collagen *(1–3)*. *In vivo*, the results are not so convincing, at least as far as the actual mechanism of inhibitory effect is concerned. What I have in mind could be best illustrated by the following examples derived from my own experimental work.

Liver fibrosis induced by injecting mice with silica is significantly reduced by simultaneous administration of a divalent iron chelator, 1,10-phenanthroline, at the dose of 2 mg/100 g body weight injected i.p. three times weekly (Table 1). It was assumed that collagen was inhibited by interference with the activity of prolyl hydroxylase by blocking the essential iron cofactor *(4)*.

Rats fed a diet containing 0.3% ethionine develop at different times first necrotic, then fibrotic lesions in the liver, and later hepatomas. Several biochemical and morphologic features of ethionine liver toxicity are almost prevented by simultaneous administration of 1,10-phenanthroline at a similar dose given above (Table 2) *(5,6)*. This finding by itself alerted us to study the actual mechanism of 1,10-phenanthroline action since not only collagen but various biochemical reactions were affected by this chelating agent. The final blow came by the finding that even the nonchelating analog of 1,10-phenanthroline, i.e., 1, 7-phenanthroline, is protective against ethionine chronic toxicity *(6)*. This led us to measure the activity of prolyl hydroxylase in various organs as well as in granuloma tissues of rats treated *in vivo* with 1,10-phenanthroline. In no single case did we find an inhibition of this enzyme or any evidence on the presence of underhydroxylated collagen *(7)*. We found, however, that single or few doses of this drug inhibit the activity of a microsomal drug-oxidizing enzyme, while repetitive administration of this conjugated aromatic molecule leads to the activation and proliferation of smooth endoplasmic reticulum, which is the site of drug metabolism *(7, 8)*. It has also been shown that several chelating agents inhibit the development of tissue injury by blocking iron involved in lipid peroxidation,

Table 1. Effect of 1,10-phenanthroline treatment on liver fibrosis induced by intravenous administration of quartz to mice.[a]

Parameter studied	Controls no SiO_2	SiO_2 only	SiO_2 + 1,10-Phenanthroline
Collagen			
Total hydroxyproline, mg/liver	0.18 ± 0.07	0.63 ± 0.17[b]	0.17 ± 0.06
C^{14}-hydroxyproline synthesis, cpm/liver	—	1138 ± 185[b]	507 ± 93
Noncollagenous proteins			
Total proline mg/liver	—	4.48 ± 0.84	3.75 ± 0.75
C^{14}-proline incorporation, 10^{-3} × cpm/liver	—	169 ± 22	140 ± 14.5

[a] To mice (H strain, 20 g body weight), within a period of 1 week, two intravenous doses of quartz dust (5 mg each, size 5 μm) were administered in a 0.5 ml saline solution. Phenanthroline was given subcutaneously twice weekly at a dose of 0.5 mg per mouse. After 3 months, C^{14}-proline was administered intraperitoneally at a dose of 6.98 μCi/mouse, and after 4 hr the animals were killed. The 0.2*M* NaCl extract from the liver homogenate was discarded; from the residue, all collagen proteins were extracted with boiling trichloroacetic acid, and the amount and activity of collagen hydroxyproline determined. In the washed precipitate, the content and activity of noncollagenous proline were determined. Variability of the data is given by mean ± S.D.

[b] Statistically significant at $p<0.001$ *(4)*.

Table 2. Effect of 1,10-phenanthroline on the content and synthesis of collagenous and noncollagenous proteins in ethionine-induced liver injury in rats. [a]

Parameter studied	Control	Ethionine	Ethionine ± 1,10-phenanthroline
Collagen			
Total *hydroxyproline, mg/liver*	0.77 ± 0.04 [b]	1.39 ± 0.13 [b]	0.75 ± 0.06
^{14}C-Hydroxyproline synthesis, 10^{-2} × cpm/liver	—	84 ± 2.7 [c]	16.0 ± 1.0
Noncollagenous proteins			
Total protein, mg/g dry fat-free liver	90 ± 4	883 ± 4	901 ± 2
^{14}C-proline incorporation, 10^{-3} × cpm/μmole proline	7980 ± 122 [c]	6695 ± 172 [d]	8757 ± 180

[a] CFN female rats were fed a diet containing 0.3% DL-ethionine for 34 days. We administered 1,10-phenanthroline (2 mg/100 g body weight) three times weekly subcutaneously. Variability is given by mean ± S.E. *(5,6).*
[b] Significant al $p<0.01$.
[c] Significant at $p<0.001$.
[d] Significant $p<0.05$.

which is one of the mechanisms labilizing various biomembranes *(9).*

In vivo, in animals either deficient in ascorbic acid *(10)* or treated with iron-chelating agents, the formation of underhydroxylated collagen has never been demonstrated. Thus, it seems that the principle of selectivity and specificity is not valid for whole animals. Furthermore, in work with fibrosis, it should be understood that 15%–20% inhibition of the collagen content in the lesion, despite being statistically significant, may not be clinically important. The clinician needs a substantial inhibition of collagen.

Finally in the search for methods specifically inhibiting collagen, Prockop and his co-workers *(1)* have done exciting work demonstrating that in *in vitro* systems, the incorporation in the collagen polypeptide of some proline analogs such as 1-azetidine-2-carboxylic acid, 3,4-dehydro-l-proline, *cis*-4-hydroxy-l-proline, etc., blocks collagen systhesis. These compounds are erroneously picked up by proline-tRNA and incorporated into collagen polypeptide chain. They are not hydroxylated, however, because of the substrate specificity of proline or lysine hydroxylase and because of eventual unavailability of the group to be hydroxylated. Thus, glycosylation and collagen secretions are also inhibited. It certainly works well *in vitro; in vivo,* the results are either unconvincing or conflicting. The near future will certainly show the validity of this *method (53, 54).*

Thus, the approach to inhibit collagen by interfering with its hydroxylation *in vivo* does not seem very promising at this moment.

Interference with Collagen Maturation

Interference with collagen maturation offers more encouragement. A large variety of so-called lathyrogens exist which are competitive inhibitors of lysyl oxidase (Fig. 2). The typical and most studied substance is β-aminopropionitrile (BAPN), which certainly *in vivo* at a certain dose significantly inhibits the formation of crosslinks between collagen molecules. It has been stressed that accumulation of large volumes of collagen in most tissues would not lead to functional disorders since such a collagen is part of the loose connective tissue. What makes the collagen scar a danger to life is the polymerization of the collagenous mass leading to a tough, rigid scar, causing strictures. At this time, "selective interference" with crosslinking appears to be "the most practical method for controlling physical properties of newly synthesized scar tissue" *(11).* Administration of BAPN in controlled doses prevents joint stiffness during immobilization, minimizes esophageal stricture following lye burns, blocks

fibrous adhesion formation, reduces tensile strength of healing wounds, increases regeneration of liver parenchyma in experimental cirrhosis, and inhibits the development of lung silicosis *(12)*. Treatment of silicotic rats with BAPN significantly lowered the lung weight and its collagen content while increasing the animals' body weights. Although Levene et al. *(12)* concluded that control of the silicotic fibrotic process is a feasible goal, nobody has expanded on this topic using more sophisticated methods of analysis.

It was found that BAPN inhibits only collagen crosslinking and does not affect collagen synthesis (Fig. 3). Probably, increasing the pool of soluble forms of collagen will increase their degradation since soluble forms are more accessible to digestion by collagenase, eventually by other proteases.

Our Division has been very much involved in studying the usefulness of BAPN as a therapeutic modality *(11)*. At this meeting, I would just like to mention our most recent finding using immobilized BAPN in the inhibition of collagen crosslinks. Principally, we were interested in local administation of BAPN. This low molecular weight drug, however, diffuses quickly through tissues and is also quickly metabolized by tissue monoaminooxidases. To slow down both of these effects, we linked BAPN to a large polymer having a molecular weight of about 5.0×10^5 daltons. This drug, after a single local injection into granuloma tissue, inhibited lysyl oxidase for the next 12 days, thus preventing the polymerization of collagen *(13)*. This method needs more experimentation. Still, we are very encouraged by our preliminary results.

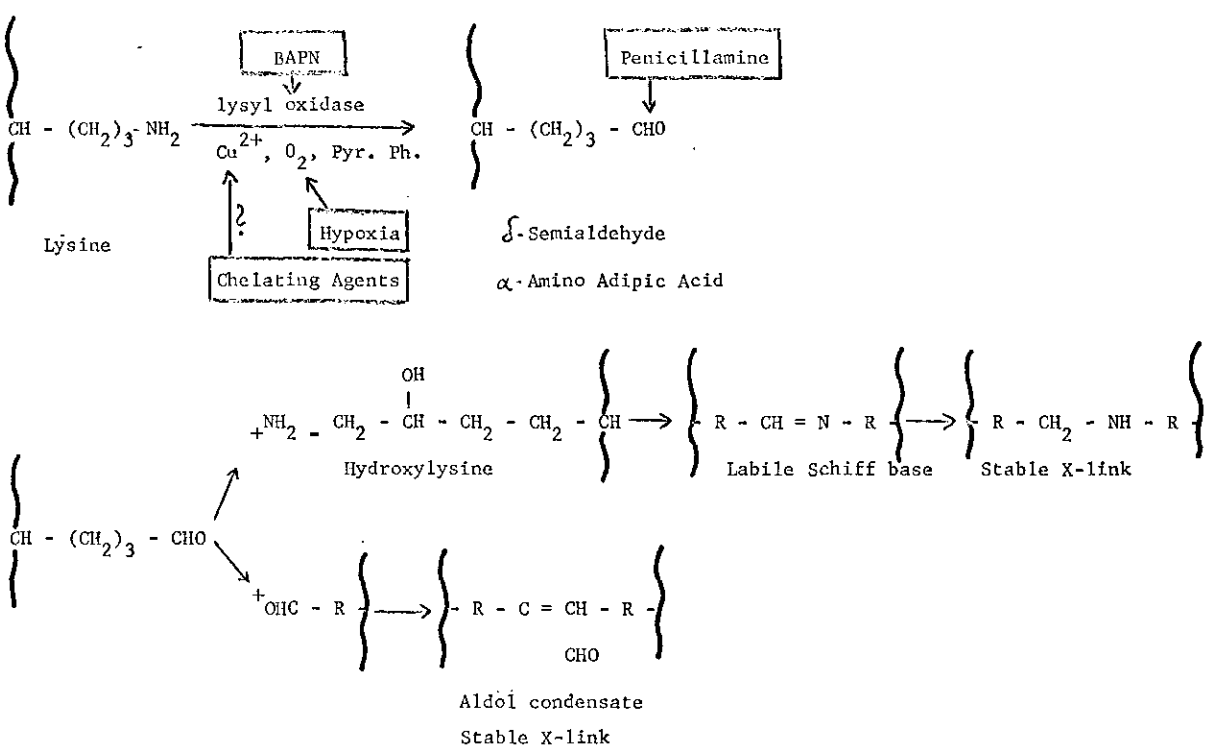

FIGURE 3. Control of covalent crosslinks synthesis in collagen. Lysyl oxidase is oxidatively desaminating some ϵ-NH_2 of lysine of hydroxylysine in collagen. The formed aldehydes interact with aldehyde of another chain under the formation of stable aldol condensate, or they react with another ϵ-NH_2-free group to form Shiff-base linkage. Only after reduction does this become stable covalent links. BAPN (β-aminopropionitrile) is a competitive inhibitor of lysyl oxidase. Penicilliamine interacts with aldehyde groups. Both reactions thus prevent the formation of crosslinks. Hypoxia affects crosslinking. So far, no evidence has been submitted indicating the direct interaction of chelating agents with copper ions in lysyl oxidase. Pyr. Ph. stands for the pyridoxal phosphate cofactor.

Oxygen and Fibrosis

Although low oxygen concentration (<2% O_2) in *in vitro* systems inhibits the hydroxylation of collagen polypeptide, this reaction was not effected in granuloma tissue of rats exposed for 11 weeks to high-altitude hypoxia *(3, 14)*. On the contrary, we observed the enhancement of collagen synthesis and accumulation, while noncollagenous protein synthesis was inhibited. Allow me to speculate on the importance of hypoxia in fibrogenesis and on the reasons for the stimulatory effect of hypoxia on fibrogenesis (Fig. 4).

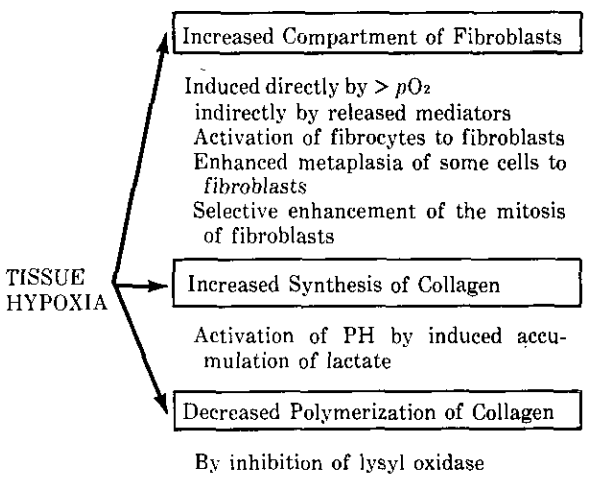

FIGURE 4. Speculative scheme on the effect of hypoxia on fibrogenesis. Local tissue hypoxia is reflected in a low pO_2. PH stands for prolylhydroxylase.

In any form of fibroproliferative inflammation at a certain time of development, a local tissue hypoxia develops. As indicated in Table 3, low oxygen may directly or indirectly affect the life of fibroblast population within a certain tissue.

The growth of new capillaries in the injured tissue proceeds in the direction of the lowest pO_2 gradient. As the capillary growth could be considered as a connective-tissue reaction (formation of basement membrances by fibroblasts which follow the endothelial cell proliferation), it would indicate increased activity of some cells in a lower pO_2 *(15)*.

Accumulation of lactate in hypoxia tissue may stimulate collagen synthesis by fibroblasts since lactate has been shown to activate the inactive forms of prolyl hydroxylase *(16)*.

Table 3. Effect of α-tocopherol on hemolysis of erythrocytes induced by quartz. [a]

Sample	Hemolysis, % of total		
	0	30 min	60 min
Ery in buffer (27°C)	1.9	22.5	42.6
Ery + 5 mg SiO_2	10.5	92.0	95.0
Ery + SiO_2 + α-tocopherol	7.4	46.0	56.4
Ery + α-tocopherol	11.2	85.0	87.0

[a] Dog erythrocytes, washed twice in Tris (0.01*M*)–NaCl (0.14*M*) buffer, pH 7.35 at 27°C were incubated at 27°C under slight shaking for 30 and 60 min. After spinning, the hemolysis was read at 540 nm. The total hemolysis for each sample was determined by adding 0.1% Triton X-100. Silica particles (1 μm) were monodisperse and contained 99.5% pure SiO_2 (Chvapil; unpublished results).

Nonspecific Interference with Abnormal Collagen

Nonspecific interference with abnormal collagen accumulation involves methods controlling any step in the inflammatory process before the fibroblast receives the message to get activated and to produce more collagen.

Thus, inhibition of the formation of connective tissue activating peptides formed in the injured tissue *(17, 18)* or "macrophage factor" as indicated by Heppleston and others *(19, 20)* may be of importance. The damage to macrophages by lysing the lysosomes by silica has been considered a key role in the process of silicotic fibrogenesis *(21–23)*. The stabilization of the lysosome membrane therefore seems a logical and interesting possibility.

In this connection, I would like to challenge two aspects of the commonly accepted concept on the role of macrophages and the mechanism of lysosomal damage in silicosis or asbestosis.

The propagation of the death of macrophages by their contact with silica is the generally accepted view on the mechanism of collagen-synthesis stimulation is silicotic lesions. It has also been established that the magnitude of fibrosis correlates with the extent of necrosis in a certain tissue. As shown by Simpson and Ross *(24)* and by Ross *(25)*, the depletion of neutrophils by injecting the animals with a

specific antineutrophil serum did not modify the dynamics of events during the development of reactive granuloma tissue. Still, there were no neutrophils present within the 10-day studies of granuloma tissue at any of the times examined. Recent evidence indicates, however, that depletion of macrophages in skin wound granulation tissue by antimacrophage serum *(25)* results in inhibition of collegan accumulation in the healing wound. This offers an exciting possibility to use this method to interfere with the role of macrophage in the development of silicotic fibrosis.

The lysis of the membrane of the macrophage phagosomes by silica has been reviewed by Allison *(23)* and described as a result of the formation of hydrogen bonds between silicic acid and components of the membrane, resulting in labilization of the whole structure *(21–23)*. I wonder if silicic acid is involved in the labilization of the membranes since in the case of the erythrocyte test, the hemolysis is enhanced almost instantaneously when the cell comes in contact with washed silica particles (Table 3). There is, however, an indication that silica enhances the level of lipid peroxidation in the exposed tissue *(26)*. In more concrete terms, all biomembranes contain a substantial amount of polyunsaturated fatty acids (PUFA), arachidonic acid (20:4) being the most abundant. PUFA are highly susceptible to nonenzymatic and to enzymatic oxidations. All forms of lipid peroxidation require iron and possibly copper ions *(9)*. Once the oxidation of PUFA is initiated (by an oxidant, a redox system, a free radical, or any form of radiation energy), it propagates under the formation of peroxides and hydroperoxides. There are several known substances within the biological structures which keep the process of lipid peroxidation in check. Among these antioxidants are GSH, ascorbic acid, vitamin E, and enzymes degrading peroxides or hydroperoxides. These are of great biological importance, though their effect is still not completely understood *(27, 28)*.

One important aspect of lipid peroxidation relates to its dependence on systems transporting electrons. For instance, NADPH-oxidation in microsomes, being the first step in drug-oxidation systems, is linked to peroxidation of lipids *(29)*. Several lines of investigation have provided evidence that polymorphonuclear leucocytes or macrophages, while involved in phagocytosis, increase their metabolism. Specifically, an activation of NADPH oxidation by NADPH oxidase was demonstrated *(30)*. This reaction by itself could explain the labilization of lysosomes related to oxidation-linked lipid peroxidation of NADPH. Another consequence is the shortening of the life span of exposed phagocytosing cells. Thus, the labilization of the lysosomal membrane by silica particles and eventually asbestos fibers may not be necessarily linked to hydrogen bonding by released silicic acid *(21)* but simply to the phenomenon of phagocytosis. However, there is a great difference in the cytotoxicity of fibrogenic and inert dust. An additive factor is therefore needed to explain the higher labilization capacity of fibrogenic dust. It may be related to the old Evans hypothesis on piezoelectricity of silica crystals *(31, 32)*, revived with modifications in the work of Hoenig et al. *(33)* and Robock *(34)*, in which the emission of electrons from the edges of tetrahedrons of silica crystals, or the exchange action in the form of charge-transfer between the silica and the cell membrane, represents in the light of the lipid-peroxidation concept the initiator of the whole process terminating in the deterioration of various biomembranes. Some evidence on the redox activity of the quartz surface has been presented by Marasas and Harington *(35)* who demonstrated *in vitro* hydroxylation of proline by silica powder. This phenomenon seems to be related to free-radical hydroxylation, possibly by a mechanism suggested by Bhatnagar et al. *(36)*.

Mechanism of Cytoxicity of Silica Particles

Below I would like to briefly summarize our preliminary data indicating that the labilization of the membrane of erythrocytes exposed to silica particles may be related to the induction of lipid peroxidation of PUFA constituents of the membrane. I will also present some results pointing to the great importance of trace elements in the control of biomembrane stability and integrity. It is obvious that this last aspect may be of great importance in our discussion on the mode of action of asbestos fibers.

Hemolysis of Erythrocytes

Hemolysis of erythrocytes induced by silica or some other particular dust was used in the past by several authors to study various aspects of the cytotoxicity of industrial dust. The extent of hemolysis in the present of silica is several times higher than in a control sample of washed erythrocytes. Addition of α-tocopherol to this sytem significantly reduced hemolysis by silica; but α-tocopherol by itself was a hemolytic agent (Table 3). α-Tocopherol is known as a free-radical scavanger, a substance reducing the lipid-peroxidative changes in various biomembranes *(27, 28)*. In our system, however, the inhibition of hemolysis could be explained by the eventual interaction of α-tocopherol with the silica surface, a mechanism postulated for several other drugs interfering with silica-induced hemolysis (polyvinyl pyridine *N*-oxide).

To test the lipoperoxidative hypothesis of the silica effect on biomembranes, we measured two parameters reflecting the peroxidation of lipids: the degradation product of peroxides, malondialdehyde (MA), and the fluorescence product formed by the conjugation of MA with other macromolecules *(37)*.

The formation of MA in silica-treated erythrocytes as compared to control samples incubated at 27°C for various time intervals showed a kinetics, indicating a higher turnover of MA in silica–erythrocyte systems (Table 4). There was more fluorescent product formed in the silica-incubated sample. The amount increased with an increase in the time of incubation (Fig. 5). Finally, the gas chromatographic analysis of the profile of fatty acids of erythrocyte ghosts prepared from the silica-treated sample showed characteristic changes in PUFA, mainly in the content of arachidonic acid, again indicating an enhanced lipid peroxidation.

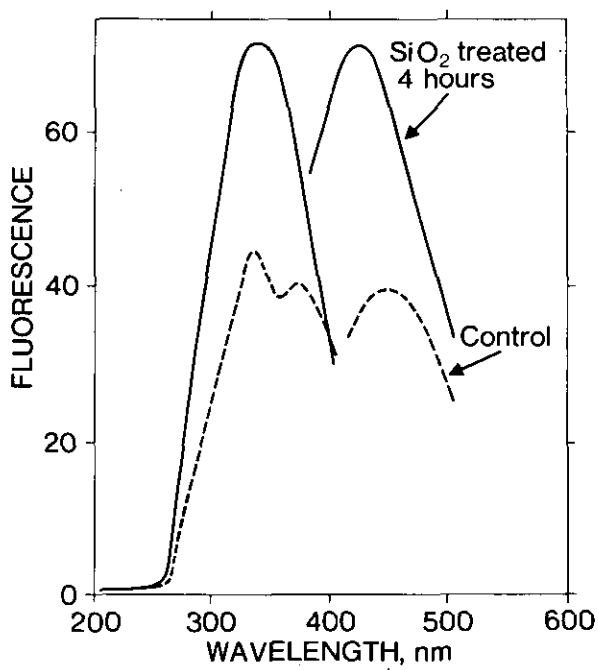

FIGURE 5. Formation of fluorescent product in erythrocytes exposed to silica. Cells incubated for 4 hr with silica were extracted with chloroform–methanol (2:1). Chloroform extract was subjected to fluorescence spectroscopy studies (Chvapil et al., unpublish results).

Table 4. Malondialdehyde formation in erythrocytes incubated with silica particles. [a]

Time of incubation (27°C, shaking), min	Malondialdehyde, ΔOD/sample	
	Control	SiO_2-treated
0	0.132	0.152
5	0.145	0.170
10	0.157	0.207
20	0.160	0.160
30	0.195	0.170
60	0.220	0.170
120	0.210	0.160

[a] 0.5 ml of washed erythrocyte pellet was suspended in 2 ml of Tris–NaCl isoosmotic buffer, pH 7.35, and incubated at 27°C under shaking without or with quartz particles (1 μm), 5 mg/sample. Reaction stopped with 30% trichloroacetic acid. The data show one characteristic experiment (Chvapil, unpublished results).

We believe that all these results indicate that silica induces lipid peroxidation at the membrane level by a still unknown mechanism. Thus, the labilization of lysosomes of macrophages by silica may be the result of the same mechanism. If this concept proves to be correct for silica and other industrial dust, the practical implication of this finding may lead to the evaluation of antioxidants, free-radical scavangers, in the control of the fibroproliferative inflammatory form induced by industrial dust, and specifically by silica (Chvapil et al. unpublished results, details will be published elsewhere).

Effect of Zinc

Effect of zinc on the viability of macrophages exposed to silica was studied in two groups of mice fed a standard diet and injected intraperitoneally, either with saline or with zinc chloride in the dose 0.1 mg/mouse. Macrophages isolated after thioglycolic-acid administration were incubated with quartz (1 mg/ml) for various time intervals. Samples of freshly isolated macrophages showed 85% viability. Exposure to silica (1 μm in size for 30 min at 37°C) decreased the viability index to 56%. Macrophages isolated from mice treated with zinc were significantly protected against the cytotoxic effect of silica (79% viable cells) (Fig. 6). Why should zinc protect the cells against

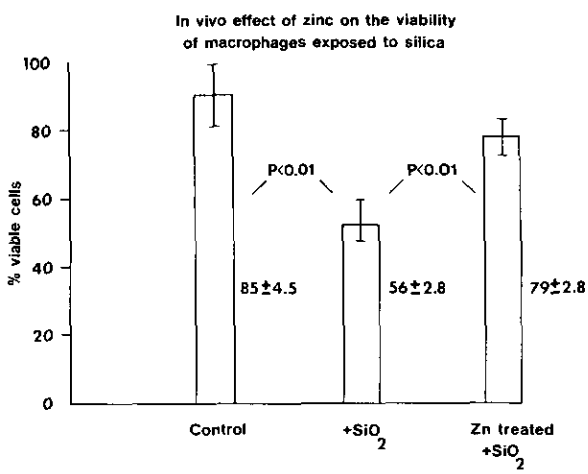

FIGURE 6. *In vivo* effect of zinc on the viability of macrophages exposed to silica. Peritoneal macrophages from control or zinc-treated mice were exposed for 30 min to SiO_2 (1 μm in size) at 37°C. The percentage of viable cells in each group is given by mean ± S.E. *(38)*.

some cytotoxic agents? I believe that the answer to this question is given by another of our experiments *(38)* in which we showed that zinc inhibits the phagocytosis of *Staph. albus* and at the same time inhibits K and Na-activated ATPase (Table 5). A similar inhibition of ATPase by some metals in decreasing orders has been shown by Mustafa et al. *(39)* and Donaldson et al. *(40)*. Since one of the functions of this enzyme has been implicated in the role in cell activities related to the functional state of the membrane such as mobility, ruffling,

Table 5. Effect of *in vivo* administration of zinc on the phagocytosis of *Staphilococcus albus* by macrophages.

	Control	Low Zn [a]	High Zn [b]
Phagocytosis index, in % of control [c]	100 ± 5.7	62.8 ± 5.2	48.7 ± 7.6
Rate of phagocytosis [d]	100 ± 1.8	73.6 ± 4.0	79.7 ± 7.6

[a] 0.05 mg $ZnCl_2$/mouse/day, i.p. for 6 days.
[b] 0.25 mg $ZnCl_2$/mouse/day, i.p. for 6 days.
[c] Refers to number of bacteria per macrophage.
[d] Refers to percentage of cells showing phagocytosis in 60 min.

engulfing, and phagocytosis, it seems that silica is simply less toxic to zinc-treated macrophages because it is not so actively phagocytosed by the cell.

Metals and Tissue Injury

In the last part of my presentation, I would like to review some newer aspects on tissue injury as a phenomenon related to labilization or disintegration of various biomembranes. A membrane is a structural composite of lipids and proteins, some of them being integral parts of the membrane structure (intrinsic) and some being macromolecules linked to the membrane (extrinsic). It is becoming ovious that the integrity of any cell biomembrane depends on a certain proportion of SS−SH groups as well as on the functional state of PUFA, mainly of phospholipid moiety. Oxidation of both SH and lipids affects membrane integrity. Metal redox systems, such as Fe^{2+}/Fe^{3+}ions stimulate lipid peroxidation *(41)*. Iron functions as a catalyst in both enzymic and nonenzymic forms of lipid peroxidation, as indicated by EDTA-inhibition studies *(41, 42)*. In rats infused continuously with $CaNa_2$ EDTA (3 mmole/kg/24 hr), the capacity of various tissues to peroxidize PUFA is abolished, the addition of minute amounts of iron restores the activity of the liver to produce MA. In this connection, I consider interesting Harington's observation *(43)* that as bestos fibers extracted with EDTA lost their cytotoxicity. This finding certainly deserves a detailed investigation. The effect of metals on the activity of various enzymes has been stressed and reviewed *(44, 45)*. I would like to point to the role of metals in the control of bio-

membrane integrity by interfering with mechanisms regulating such a stability. This refers to the direct interaction of the metal with membrane constituents, and with enzymes affecting membrane components and the process leading to the induction of lipid peroxidation, etc. Some of these aspects have been reviewed *(46—48)*.

The lysosomal theory of tissue injury proved to be of paramount importance in understanding the dynamics of events after the effect of physical, chemical, or biological noxious agents. In our experimental work, we showed that zinc stabilizes liver lysosomes *in vitro (49, 50)* and *in vivo (50)*. The finding that unsaturated complexes of zinc-8-hydroxyquinoline (1:1 or 1:2) which do not permeate cell membranes and which are bound exclusively to the membrane surface are even more effective than zinc alone indicates that the reaction stabilizing lysosomes is confined to the surface of the organelle.

Zinc is not the only metal stabilizing lysosomes. Cadmium and lead at a final concentration of 1 m*M* are effective, whereas manganese and nickel are inactive in our experimental system *(50)*.

There are data showing that similar stabilization (or better prevention of labilization) of lysosomes occurs *in vivo* in situations where the integrity of lysosomes is impaired by such agents as CCl_4 or by high toxic doses of $CaNa_2$ EDTA *(42, 51)*. The necrogenic action of CCl_4 has been attributed to its metabolite, the trichloromethyl radical $CCL_3\cdot$, which initiates lipid peroxidation; the actual step affected by zinc is still unknown. We showed that NADPH-oxidation in microsomal liver fractions is inhibited by minute amounts of zinc *(48)*. Superoxide-dismutase, an enzyme degrading superoxide anion, is a zinc-containing enzyme *(52)*.

Other evidence indicating the interference of zinc with lipid peroxidation *in vivo* was presented by Radomski and Wood *(53)* and Chvapil et al. *(46)*. They showed that lung edema induced by exposing mice to hyperbaric oxygen or NO_2 can be prevented by adequate treatment of animals with zinc or manganese. Oxygen, NO_2, and O_3 are known to induce lipid peroxidation in the lung.

In summary, I believe that various metals are involved in the mechanism regulating the integrity of biomembranes. Some are labilizers, some are stabilizers, but the actual concentration of a certain metal as well as the whole microenvironment may determine the nature of the effect of a certain metal ion on a biomembrane.

Acknowledgements

This work was supported by National Science Foundation grant GB 33928 and Public Health Service grants AM 10447, AM 15460-02, ES 00790, and AM 16489.

NOTE ADDED IN PROOF: Since this paper was presented, several important papers were published *(55—57)* indicating in greater detail the presence of fibroblast stimulating factors in activated macrophages and the formation of lipid peroxides in cells under the effect of silica.

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